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Cytotoxicity and Membrane Interaction of Tamoxifen as Affected by Ca^{2+} and Mg^{2+}: Use of a Bacterial Model System

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Abstract—A strain of *Bacillus stearothermophilus* was used as a model to study the interaction of tamoxifen (TAM) with the membrane and the cytostatic antiproliferative effects not related to estrogen binding. TAM inhibits the growth of *B. stearothermophilus* as a function of concentration. The supplementation of the growth medium with Ca^{2+} or Mg^{2+} partially relieves the growth inhibition by TAM, allowing growth at TAM concentrations that fully impair growth in the basal medium. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and of its propionic acid derivative (DPH-PA) reveals opposite effects induced by TAM and Ca^{2+}. The addition of Ca^{2+} to liposomes of bacterial lipids promoted physical ordering as opposed to disordering induced by TAM. Thus, it is predictable that growth impairment induced by TAM is mediated through perturbations at the membrane level.

Keywords: calcium; magnesium; tamoxifen; *Bacillus stearothermophilus*; bacterial growth; membrane physical effects.

Abbreviations: DPH = 1,6-diphenyl-1,3,5-hexatriene; DPH-PA = 3-*p*-(6-phenyl)-1,3,5-hexatrienyl-phenylpropionic acid; TAM = tamoxifen; ER = estrogen receptor.

INTRODUCTION

Tamoxifen (TAM), the non-steroidal anti-estrogen widely administered to breast cancer patients, is believed to act by competing with estrogen receptors (ER). However, the existence of alternative mechanisms of action is supported by the clinical observations that some patients with ER-negative cancer cells respond to TAM and some patients with ER-positive cancer cells are not sensitive to this anti-estrogen.

Several anticancer drugs interact strongly with the cell membrane; these interactions presumably play important roles either in the antitumoral activity or in the cytotoxicity of these drugs (Balasubramanian and Straubinger, 1994; Canaves *et al*., 1991; Deliconstantinos *et al*., 1987; Wright and White, 1986).

To elucidate molecular mechanisms of TAM at the membrane level and the cytotoxic effects, a bacterium has been used as a model system (Luxo *et al*., 1996). As the structure and composition of eukaryotic membranes are comparatively complex, prokaryotic membranes are suitable models to study membrane mediated toxic effects for the following reasons: (a) basically, the bacterial membrane consists of a lipid bilayer and bacterial phospholipids are structurally similar to eukaryotic counterparts (Ratledge and Wilkinson, 1988); (b) functionally, bacterial lipids play roles similar to eukaryotic lipids (Russel, 1989), providing a fluid continuous matrix to incorporate proteins; (c) membrane proteins of bacterial cells carry out most of the cell functions which depend on the physical properties of the lipid bilayer for optimal activities (McElhaney, 1982), as in the case of eukaryotic membranes. Therefore, the drug interactions and TAM effects on the biophysical properties of membrane lipids can be extrapolated to more complex membrane systems. Very often, bacterial cells have been used as good experimental tools in modelling studies of mechanisms underlying the action of membrane-active drugs (Sikkema *et al*., 1995; Silva *et al*., 1979), offering important advantages over eukaryotic cells, namely: (a) the membrane organization is comparatively simple; (b) there are no intracellular compartments; (c) a single membrane involves the cytoplasm in Gram positive Eubacteria.

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TAM inhibits growth of *Bacillus stearothermophilus* as a function of concentration (Luxo *et al.*, 1996). Studies with fluorescent probes of fluidity 1,6-diphenyl-1,3,5-hexatriene (DPH) and its propionic acid derivative (DPH-PA), in several models, either lipid vesicles or native membranes (Custódio *et al.*, 1993; Luxo *et al.*, 1996), suggest significant structural disorder of the lipid bilayer induced by TAM.

To further clarify the physicochemical basis of TAM-membrane mediated cytotoxicity, we performed studies of TAM perturbations on growth, in the presence of Ca^{2+} and Mg^{2+} and on the physical properties of bacterial phospholipid membranes in the presence of Ca^{2+}, a natural membrane stabilizer (Jurado *et al.*, 1991; Livingstone and Schachter, 1980; Mosley *et al.*, 1976).

MATERIALS AND METHODS

The strain of *Bacillus stearothermophilus* and the conditions for its maintenance and growth have been described previously (Jurado *et al.*, 1991). TAM from a concentrated ethanolic solution was added to the growth medium, supplemented or not with 2.5 mM Ca^{2+} or Mg^{2+} chlorides, in order to obtain concentrations from 1 to 10 μM. The final concentrations of ethanol on the growth medium ranged from 1.71 to 17.1 mM for a TAM concentration of 1 to 10 μM. Growth was stopped at the beginning of the stationary phase. The cells were harvested by centrifugation at 6000 ***g*** for 10 min, washed with buffer (10 mM Tris–HCl, pH 7.0). Lipids were extracted by the method of Bligh and Dyer (1959) and quantified by measuring the amount of inorganic phosphate (Bartlett, 1959), after hydrolysis of the extracts at 180°C in 70% $HClO_4$ (Böttcher *et al.*, 1961). The polar lipids were isolated by preparative thin-layer chromatography and liposomes were prepared according to the method described elsewhere (Jurado *et al.*, 1991). Two fluidity probes were used: DPH and DPH-PA. DPH in tetrahydrofuran and DPH-PA in dimethylformamide were incorporated into liposome suspensions (345 μM in phospholipid), as previously described (Antunes-Madeira *et al.*, 1994) to give a lipid/probe molar ratio of about 400.

The fluorimetric measurements were performed with an Perkin–Elmer LS 50 computer controlled spectrofluorometer as described elsewhere by Luxo *et al.* (1996).

RESULTS

Effect of tamoxifen on the growth of B. stearothermophilus, as affected by Ca^{2+} and Mg^{2+}

B. stearothermophilus was grown at 65°C in a complex medium (basal medium) supplemented or not with Ca^{2+} or Mg^{2+} at final concentrations of 2.5 mM. According to previous results (Luxo *et al.*, 1996), the addition of TAM to the growth medium induced bacterial growth inhibition as a function of concentration. The supplementation of the growth medium with Ca^{2+} or Mg^{2+} relieved or attenuated the inhibition induced by TAM (Fig. 1). Thus, the addition of 5.0 μM TAM to the basal medium induced a significant inhibition of growth. However, in Ca^{2+} or Mg^{2+} medium, a reasonable bacterial growth was observed in the presence of this concentration of TAM. Furthermore, growth in the basal medium was completely inhibited in the presence of 7.5 μM TAM, but this concentration of TAM promoted a limited inhibition of growth when Ca^{2+} or Mg^{2+} were present.

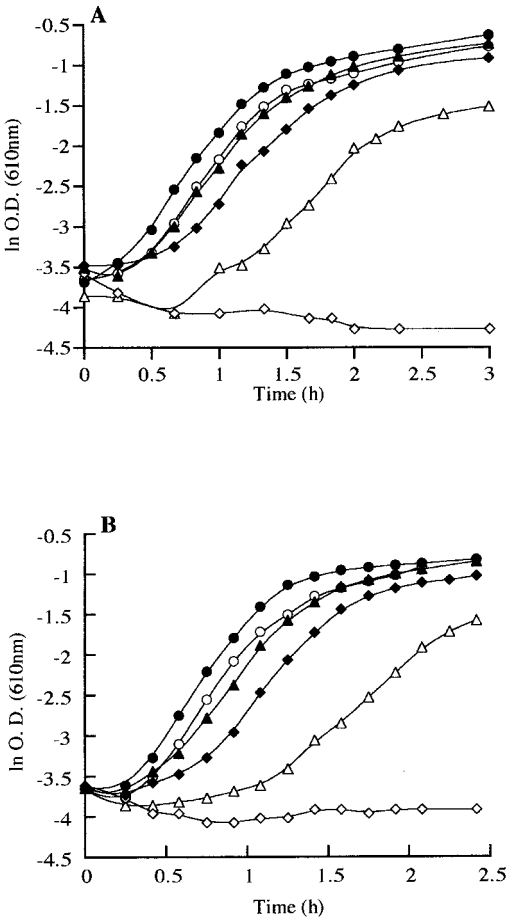

Fig. 1. Effect of tamoxifen and Ca^{2+} or Mg^{2+} ions on the growth of cultures of *Bacillus stearothermophilus* at 65°C (Optimal growth temperature). (A) Cells were grown in a basal medium (open symbols) or in a Ca^{2+}-supplemented medium (solid symbols), without (○, ●) and with 5.0 μM (△, ▲) and 7.5 μM (◇, ◆) TAM. (B) Cells were grown in a basal medium (open symbols) or in a Mg^{2+}-supplemented medium (solid symbols), without (○, ●) and with 5.0 μM (△, ▲) and 7.5 μM (◇, ◆) TAM. The results shown are typical of three (A) and two (B) separate experiments.

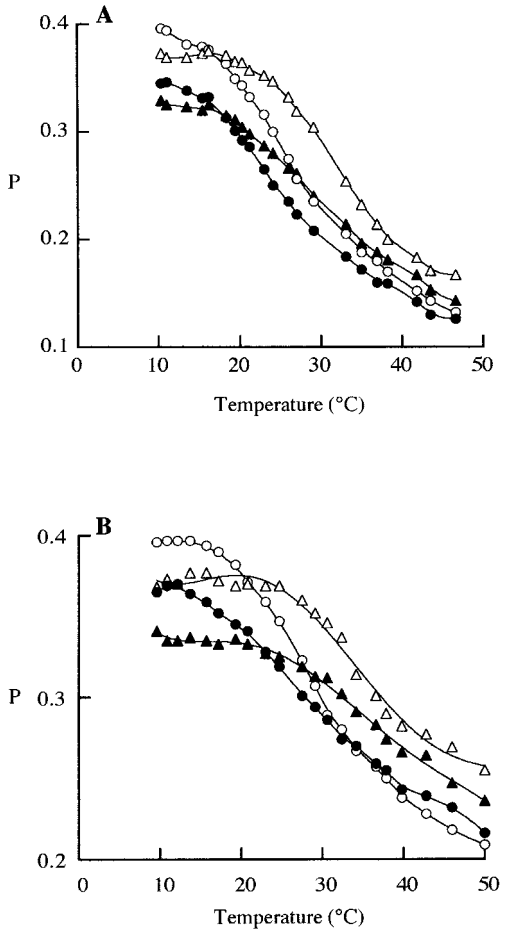

Fig. 2. Thermograms of fluorescence polarization (P) of DPH (A) and DPH-PA (B) in liposomes prepared with the polar lipids of cells grown in the basal medium, at 65°C. Control liposomes (○, ●) were prepared in a buffer without Ca^{2+}, and Ca^{2+} liposomes (△, ▲) in a buffer with 2.5 mM Ca^{2+}. Control and Ca^{2+}-liposomes were incubated without TAM (○, △) or with 50 μM TAM (●, ▲). The thermotropic profiles shown are typical of three independent experiments. Polarization data are average of three readings and error bars are not represented, since for most points, they are encompassed by the size of the symbols.

Physical effects of tamoxifen on bilayers of bacterial polar lipids, as affected by Ca^{2+}

The effects of TAM and Ca^{2+} on physical properties of membrane polar lipids of *B. stearothermophilus* were studied by fluorescence polarization of DPH and DPH-PA. DPH and its propionic acid derivative DPH-PA have been widely used to monitor membrane organization. DPH is localized within the hydrophobic core of the membrane and provides information on this region (Shinitzky and Barenholz, 1978). DPH-PA is anchored in close proximity to the bilayer surface, providing information on the bilayer organization close to the surface (Trotter and Storch, 1989). Thus, with these two probes it is possible to compare the relative order perturbations induced by TAM and Ca^{2+} ions in different regions across the bilayer. Figure 2 shows the thermograms of liposomes prepared with the polar lipids of *B. stearothermophilus*. Liposomes prepared with the heterogeneous mixture of bacterial polar lipids exhibit a broad transition with an amplitude of about 20°C, as detected by DPH (Fig. 2A) and DPH-PA (Fig. 2B), as previously reported (Jurado *et al.*, 1991).

According to previous findings (Luxo *et al.*, 1996), the addition of 50 μM TAM to bacterial liposomes induced a decrease in molecular packing in the gel phase and along the phase transition temperature range. This effect was detected by both fluidity probes, indicating a disorder effect induced by TAM across the bilayer. TAM disordering effects are similarly induced in bacterial lipid dispersions prepared with or without Ca^{2+}, that is, in control and Ca^{2+} liposomes. In agreement with earlier work (Jurado *et al.*, 1991), Ca^{2+}-supplemented liposomes exhibited a significant order increase along the transition temperature range and in the fluid phase although displaying slight disordering in the gel phase of the lipids. Although the main effect of TAM was the disordering of both types of liposomes (Ca^{2+} liposomes and control liposomes), the general order of Ca^{2+} liposomes remained higher than that of control liposomes in the presence of TAM over all the transition temperature range and in the fluid phase. The findings with DPH and DPH-PA suggest an effective counteraction of the disordering effects of TAM by Ca^{2+}.

DISCUSSION

B. stearothermophilus has been used as a model to clarify the antiproliferative action of tamoxifen putatively related with drug–membrane interaction (Luxo *et al.*, 1996). This bacterium is a useful methodological tool to study drug toxicity mediated by physical perturbations at the membrane level, since growth of this bacterium is strongly affected by agents (drugs, ions and temperature) which induce alterations of the physical properties of membrane lipids (Jurado *et al.*, 1991; Luxo *et al.*, 1996). In agreement with previous data (Luxo *et al.*, 1996), it was observed that the addition of increasing concentrations of TAM to the growth medium (basal or Ca^{2+}/Mg^{2+} supplemented) induced a progressively negative impact on all measured growth parameters, namely the lag time, the specific growth rate and the bacterial yield, indicating growth inhibition promoted by the cytostatic. The alteration of bacterial growth parameters is significantly extensive in a basal medium, as compared with Ca^{2+} or Mg^{2+}-supplemented medium, reflecting a protection by Ca^{2+} and Mg^{2+}. Stimulatory effects promoted by Ca^{2+} and Mg^{2+} have also been reported for a *Pseudomonas putida* strain growing in the presence of repressive solvents (Inoue *et al.*, 1991).

Consistently, fluorescence polarization of DPH and of its propionic acid derivative DPH-PA showed that the addition of Ca^{2+} ions (2.5 mM) effectively compensates for the disordering effects promoted by TAM in bacterial lipid membranes. Ca^{2+} stimulatory effect on growth is presumably due to a direct Ca^{2+} interaction with the membrane phospholipids, inducing an increased structural order on the bilayer. Very often, interaction of lipophilic compounds with the phospholipid bilayer of microorganisms leads to dramatic changes in the membrane structure, consequently affecting growth and viability (for a review see Sikkema *et al*., 1995). As the bacterial cytoplasmic membrane plays a crucial role in signal and energy transduction (Stock *et al*., 1990; Trumpower and Gennis, 1994), regulation of the intracellular environment (Booth, 1985), transport of solutes (Kabak, 1972) and other vital cellular functions, it is expected that perturbations of membrane structure and function by drugs will end in severe damage and inhibition of cell growth and viability. Therefore, membranes of microorganisms, for example, *B. stearothermophilus*, may be particularly useful for modelling toxicity of drugs and other compounds of industrial interest.

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